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ORIGINAL PAPER

Exposure of Cultured Astrocytes to Menadione Triggers Rapid Radical Formation, Glutathione Oxidation and Mrp1-Mediated Export of Glutathione Disulfide

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Abstract
Menadione (2-methyl-1,4-naphthoquinone) is a synthetic derivative of vitamin K that allows rapid redox cycling in cells and thereby generates reactive oxygen species (ROS). To test for the consequences of a treatment of brain astrocytes with menadione, we incubated primary astrocyte cultures with this compound. Incubation with menadione in concentrations of up to 30 µM did not affect cell viability. In contrast, exposure of astrocytes to 100 µM menadione caused a time-dependent impairment of cellular metabolism and cell functions as demonstrated by impaired glycolytic lactate production and strong increases in the activity of extracellular lactate dehydrogenase and in the number of propidium iodide-positive cells within 4 h of incubation. In addition, already 5 min after exposure of astrocytes to menadione a concentration-dependent increase in the number of ROS-positive cells as well as a concentration-dependent and transient accumulation of cellular glutathione disulfide (GSSG) were observed. The rapid intracellular GSSG accumulation was followed by an export of GSSG that was prevented in the presence of MK571, an inhibitor of the multidrug resistance protein 1 (Mrp1). Menadione-induced glutathione (GSH) oxidation and ROS formation were found accelerated after glucose-deprivation, while the presence of dicoumarol, an inhibitor of the menadione-reducing enzyme NQO1, did not affect the menadione-dependent GSSG accumulation. Our study demonstrates that menadione rapidly depletes cultured astrocytes of GSH via ROS-induced oxidation to GSSG that is subsequently exported via Mrp1.

Keywords Astrocytes · GSSG · Menadione · Mrp1 · Oxidative stress · Radicals

Introduction

Menadione (2-methyl-1,4-naphthoquinone) is a synthetic derivative of vitamin K [1]. As a quinone, menadione can be reduced in one- and two-electron transfer reactions. The one-electron reduction of menadione generates menadione semiquinone radical, which is prone to take part in cellular redox cycling and in the formation of superoxide which can ultimately cause cell toxicity due to oxidative stress [2]. The two-electron reduction of menadione generates menadione hydroquinone which can be eliminated from cells after phase II enzyme-mediated coupling to glutathione (GSH) or glucuronic acid [2, 3] and subsequent export of the conjugates. The one-electron reduction of menadione to the unstable semiquinone is catalysed by cytochrome P450 monooxygenases, whereas the cytosolic NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) can catalyze the two-electron reduction of menadione [4], thereby preventing the formation of a semiquinone intermediate and being beneficial for cells [5].

GSH is one of the major cellular antioxidants [6–9]. It can directly react with radicals such as superoxide and delivers electrons for the reduction of peroxides by glutathione peroxidases [6]. The product of such reactions is glutathione disulfide (GSSG) which is reduced in cells to GSH in the NADPH-dependent reaction, catalyzed by glutathione reductase [6, 9].

In brain, astrocytes have a key function in the GSH metabolism and in the protection against reactive oxygen

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species (ROS) and xenobiotics [6]. Astrocytes contain GSH in millimolar concentrations [7, 10] and the ratio between GSH and GSSG is very high in unstressed cells [11, 12]. Several compounds have been reported to deplete astrocytes of GSH including alkylating substances such as iodoacetamide [13], 3-bromopyruvate [14] or dialkyl-fumarates [15]. Transient depletion of cellular GSH was also found for astrocytes exposed to peroxides [11] or catecholamines [16] due to the rapid oxidation of GSH to GSSG and subsequent export of GSSG. Predominantly responsible for the export of GSH, GSSG and GSH-conjugates from astrocytes is the multidrug resistance protein 1 (Mrp1) [17–22].

In menadione exposed cells, alterations in GSH metabolism have to be considered. The formation of superoxide during menadione redox-cycling can, in addition to directly causing oxidative damage to biomolecules, also weaken the cellular defence against oxidative stress by depleting cellular levels of GSH and NADPH [23]. In addition, during menadione hydroquinone elimination the concentration of the cellular GSH pool can be lowered by conjugate formation [2]. Finally, menadione can directly react with GSH and form a 3-glutathionyl-2-methyl-1,4-naphthoquinone [2, 24], that can also be a substrate of NQO1 and has been reported to even autoxidise faster than the unsubstituted menadione after enzymatic reduction [25].

Menadione has frequently been applied to cultured astrocytes to induce ROS-formation [26–28] and menadione is known for its toxic potential on astrocytes [29–31]. Very recently we have reported that application of 100 µM menadione to cultured astrocytes causes rapid GSH oxidation and GSSG export from viable astrocytes that was prevented by Mrp1 inhibitors [32]. In order to bring this initial observation into a broader context and to substantially extend the current knowledge on the consequences of a menadione exposure of astrocytes, we have investigated now in detail the time- and concentration-dependencies of the toxicity, the ROS formation, the GSSG accumulation and the Mrp1-mediated GSSG export from cultured astrocytes after application of menadione. In addition, we provide experimental evidence showing that ROS formation and cellular GSSG accumulation occurs during glucose deprivation already at lower micromolar concentrations of menadione and that the enzyme NQO1 is unlikely to be involved in the menadione-induced ROS formation and GSSG accumulation in astrocytes.

Materials and Methods

Material

Menadione and dicoumarol were purchased from Sigma (Steinheim, Germany) and MK571 from Biomol (Hamburg, Germany). Powder for the preparation of Dulbecco's Modified Eagle's Medium (DMEM with 25 mM glucose) and penicillin/streptomycin solution were obtained from Gibco (Darmstadt, Germany). Fetal calf serum (FCS) was purchased from Biochrom (Berlin, Germany) and dimethyl sulfoxide (DMSO) from VWR Chemicals (Darmstadt, Germany). The enzymes glutathione reductase, lactate dehydrogenase (LDH) and glutamate pyruvate transaminase were obtained from Roche (Mannheim, Germany). Other chemicals of the highest available purity were from AppliChem (Darmstadt, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Riedel-de Haën (Seelze, Germany) or Sigma (Steinheim, Germany). Sterile 24-well plates for cell culturing and non-sterile 96-well microtiter plates were purchased from Sarstedt (Nümbrecht, Germany).

Astrocyte Cultures

Astrocyte-rich primary cultures were prepared from the total brains of new born Wistar rats and cultured as previously described in detail [33]. The harvested cells were seeded in a density of 300,000 viable cells per well of 24-well plates in 1 mL of culture medium (90% DMEM, 10% FCS, 1 mM pyruvate, 18 U/mL penicillin G and 18 µg/mL streptomycin sulfate) and were cultivated at 37 °C with 10% CO_2 in the humidified atmosphere of a cell incubator (Sanyo, Osaka, Japan). The culture medium was renewed every 7th day and on the day before an experiment was performed. The cultures contain predominantly astrocytes and only minor amounts of oligodendrocytes and microglial cells [33, 34]. Experiments were performed on confluent cultures of an age between 14 and 28 days.

Cell Incubations

The consequences of an exposure of cultured astrocytes with menadione were investigated for incubations of up to 6 h with menadione in concentrations of up to 200 µM. Stock solutions of menadione were prepared in DMSO and diluted in glucose-containing incubation buffer (IB: 20 mM HEPES, 5 mM D-glucose, 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4, pH 7.4) to the final concentrations applied in the cell experiments. Within one type of experiment the final concentration of DMSO was kept constant to 0.05% (Fig. 1, 2, 3, 5, 6, 7, 8) or 0.1% (Fig. 4). For the incubations, astrocytes were washed twice with 1 mL of prewarmed (37 °C) IB and were then incubated for the given incubation periods at 37 °C in a cell incubator without CO_2 supply in 200 µL IB containing menadione and/or other compounds in the concentrations indicated. The incubation was terminated by harvesting the incubation medium and by washing the cells with 1 mL icecold (4 °C)

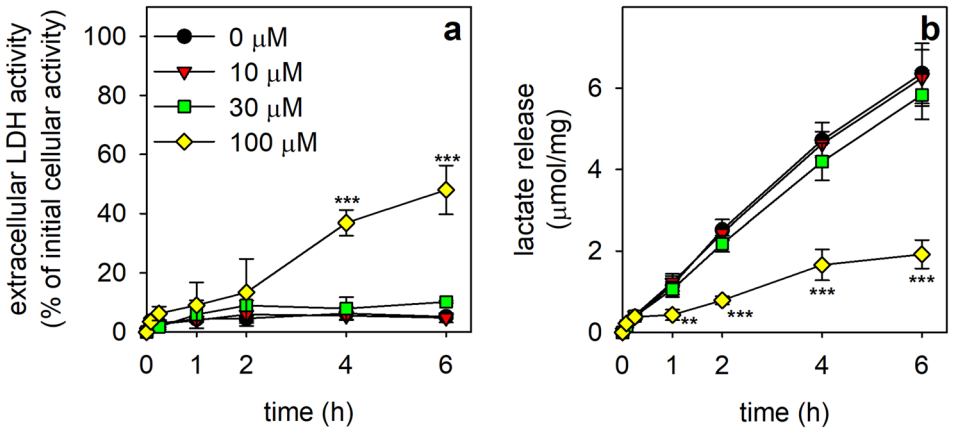


Fig. 1 Effects of menadione on the cell membrane integrity and lactate production. Cultured astrocytes were incubated without or with 10, 30 or 100 µM menadione for up to 6 h and the activity of extracellular LDH (**a**) and the content of lactate released by the cells (**b**) were determined. The initial cellular LDH activity (100%) accounted for 1777 ± 118 nmol/(min × mg) and the protein content of the cultures was 129 ± 3 µg/well. The data shown represent means ± SD of results that were obtained in experiments performed on three independently prepared cultures. The significance of differences compared to the values obtained for the control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (**p < 0.01, ***p < 0.001)

phosphate-buffered saline (PBS: 10 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4).

For some experiments the cells were deprived of glucose. For such experiments, the cultures were washed with 1 mL of prewarmed (37 °C) glucose-free IB, then preincubated for 20 min in 200 µL glucose-free or glucose-containing (5 mM) IB and then incubated for the indicated main incubation periods without or with menadione and/or other compounds in glucose-free or glucose-containing IB at 37 °C.

Quantification of Glutathione and Glutathione Disulfide

Total glutathione (GSx = amount of GSH plus twice the amount of GSSG) and GSSG in cell lysates and incubation media were determined by a microtiter-based assay as previously described in detail [33], which is based on the original cycling method published by Tietze [35]. The washed cells were lysed on ice with 200 µL 1% (w/v) sulfosalicylic acid and the lysates were used to determine GSx and GSSG. For quantification of the extracellular contents of GSx and GSSG, 20 µL of a 1:1 mixture of the harvested medium and 1% (w/v) sulfosalicylic acid were used in the cycling assay.

Tests for Cell Viability

The viability of astrocyte cultures after a given treatment was investigated by determining the extracellular activity of LDH in 10 µL medium samples and by propidium iodide staining as describe previously [33]. Extracellular LDH activity is given as percent of the initial cellular LDH activity that was determined for untreated control cells after lysis in 200 µL IB containing 1% (v/v) Triton X100. For propidium iodide staining the treated cultures were washed twice with 1 mL of prewarmed (37 °C) IB and then incubated for 15 min at 37 °C with 500 µL propidium iodide staining solution (5 µM propidium iodide plus 10 µM H33342 in IB). Subsequently, the cells were washed with IB, and fluorescence images were recorded with an Nikon Eclipse TE2000U fluorescent microscope with a DSQiMc camera and the imaging software NIS-Elements BR (Nikon, Düsseldorf, Germany) using the following filter settings for propidium iodide (excitation: 510–560 nm; emission: 590 nm; dichromatic mirror: 575 nm) and for H33342 (excitation: 330–380 nm; emission: 435–485 nm; dichromatic mirror: 400 nm). For all images shown in one multipanel figure, identical microscopic settings and image processing was applied. The fluorescence intensity of the images recorded after PI and H33342 staining was quantified using the software ImageJ and the relative PI fluorescence was calculated without any blank or background correction by normalizing the PI fluorescence to the H33342 fluorescence.

Lactate and Protein Quantification

Glycolytic activity of astrocytes was quantified by determining the extracellular accumulation of lactate in 10 µL samples of the incubation medium by a coupled enzymatic assay system with LDH and glutamate pyruvate transaminase as described previously in detail [33, 36]. The protein content of the cultures was determined according to the Lowry method [37] using bovine serum albumin as standard protein.

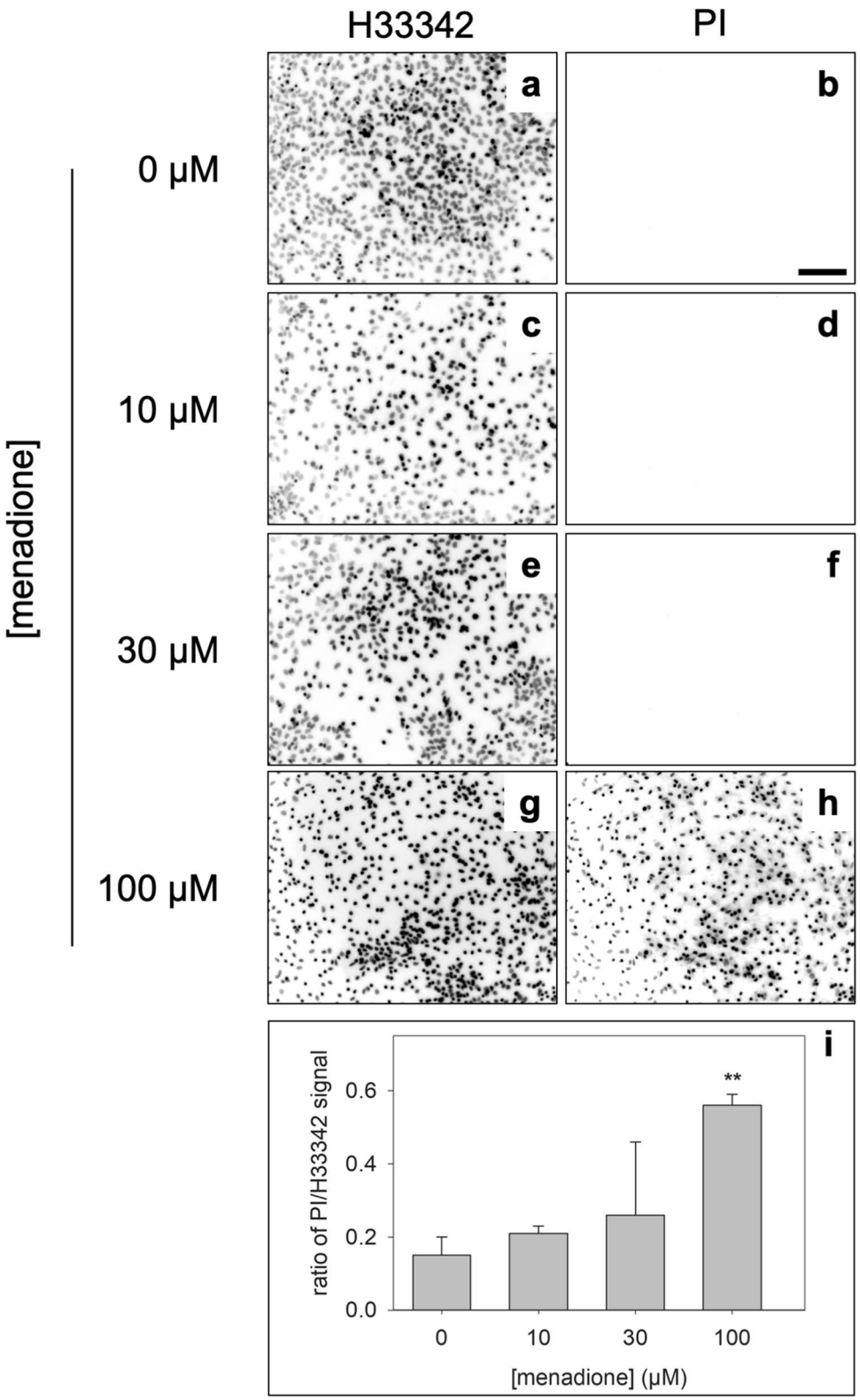


Fig. 2 Propidium iodide staining of astrocytes after menadione treatment. The cultures were incubated for 4 h with menadione in the indicated concentrations and stained with propidium iodide (PI; b, d, f, h) and Hoechst 33342 (H33342; a, c, e, g) to test for membrane permeability and to visualize the total number of cell nuclei, respectively. The scale bar in panel b represents 100 µm and applies to all panels. Panel i shows the relative PI fluorescence (ratio of the PI to H33342 fluorescence intensity signals). The data presented in panel i are means ± SD of results that were obtained in experiments performed on three independently prepared cultures. The significance of differences compared to the values obtained for the control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (**$p < 0.01$)

Test for ROS Formation

Cellular formation of ROS was investigated by visualizing the ROS-dependent oxidation of dihydrorhodamine123 to the fluorescent rhodamine123 by a modification of a published method [38]. The treated cells were washed twice with 1 mL of prewarmed (37 °C) glucose-free IB and then incubated for 30 min at 37 °C with 250 µL dihydrorhodamine123

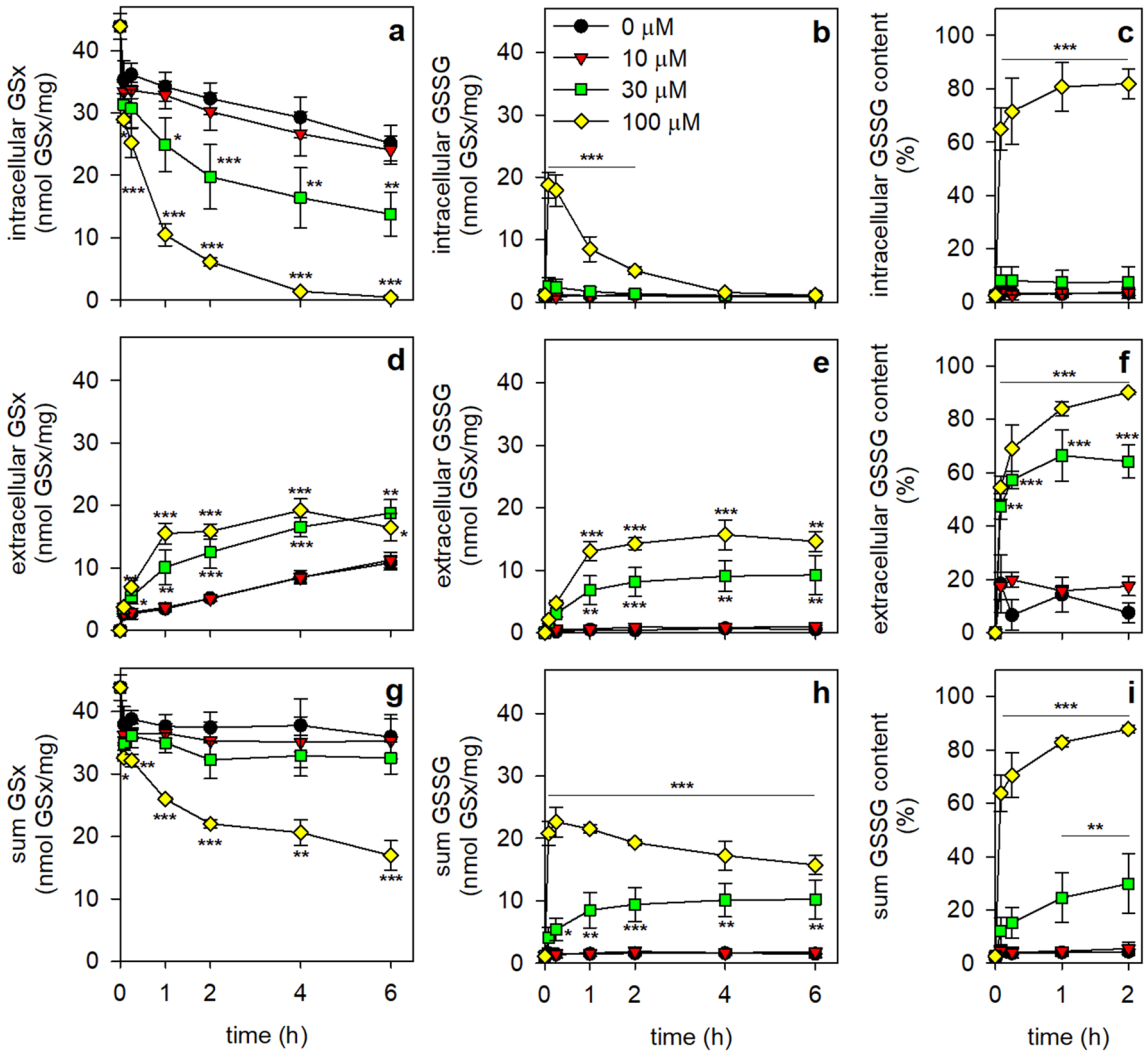


Fig. 3 Consequences of a menadione treatment on the GSx and GSSG content. Cultured astrocytes were incubated without or with 10, 30 or 100 µM menadione for up to 6 h. For the indicated time periods, the intra- and extracellular contents of GSx (**a, d**) and GSSG (**b, e**) were determined. In addition, the sum of intra- and extracellular GSx (**g**) and GSSG (**h**) was calculated. Moreover, the percental contribution of GSSG to the GSx values determined (**c, f, i**) were calculated for the initial 2 h of incubation as no loss in membrane integrity was observed for this time period (see Fig. 1). The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content was 44 ± 2 nmol/mg, the initial cellular GSSG content 1 ± 1 nmol GSx/mg and the initial protein content 129 ± 3 µg/well. The significance of differences compared to the values obtained for the control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

staining solution (5 µg/mL dihydrorhodamine123 plus 10 µM H33342 in glucose-free IB). Finally, the cells were washed twice with 1 mL prewarmed (37 °C) IB and analysed for fluorescence on the Eclipse TE2000U fluorescent microscope using the following filter settings: rhodamine123 (excitation: 465–495 nm; emission: 505–515 nm; dichromatic mirror: 505 nm) and H33342 (excitation: 330–380 nm; emission: 435–485 nm; dichromatic mirror: 400 nm). For all images shown in one multipanel figure, identical microscopic settings and image processing was applied. The fluorescence intensity of the images recorded after staining for rhodamine123 and for H33342 was quantified using the software ImageJ and the relative rhodamine123 fluorescence was calculated without any blank or background correction by normalizing the rhodamine123 fluorescence to the H33342 fluorescence.

Statistical Analysis

All quantitative data shown are means ± standard deviations of values that were obtained in experiments performed on three independently prepared astrocyte cultures.

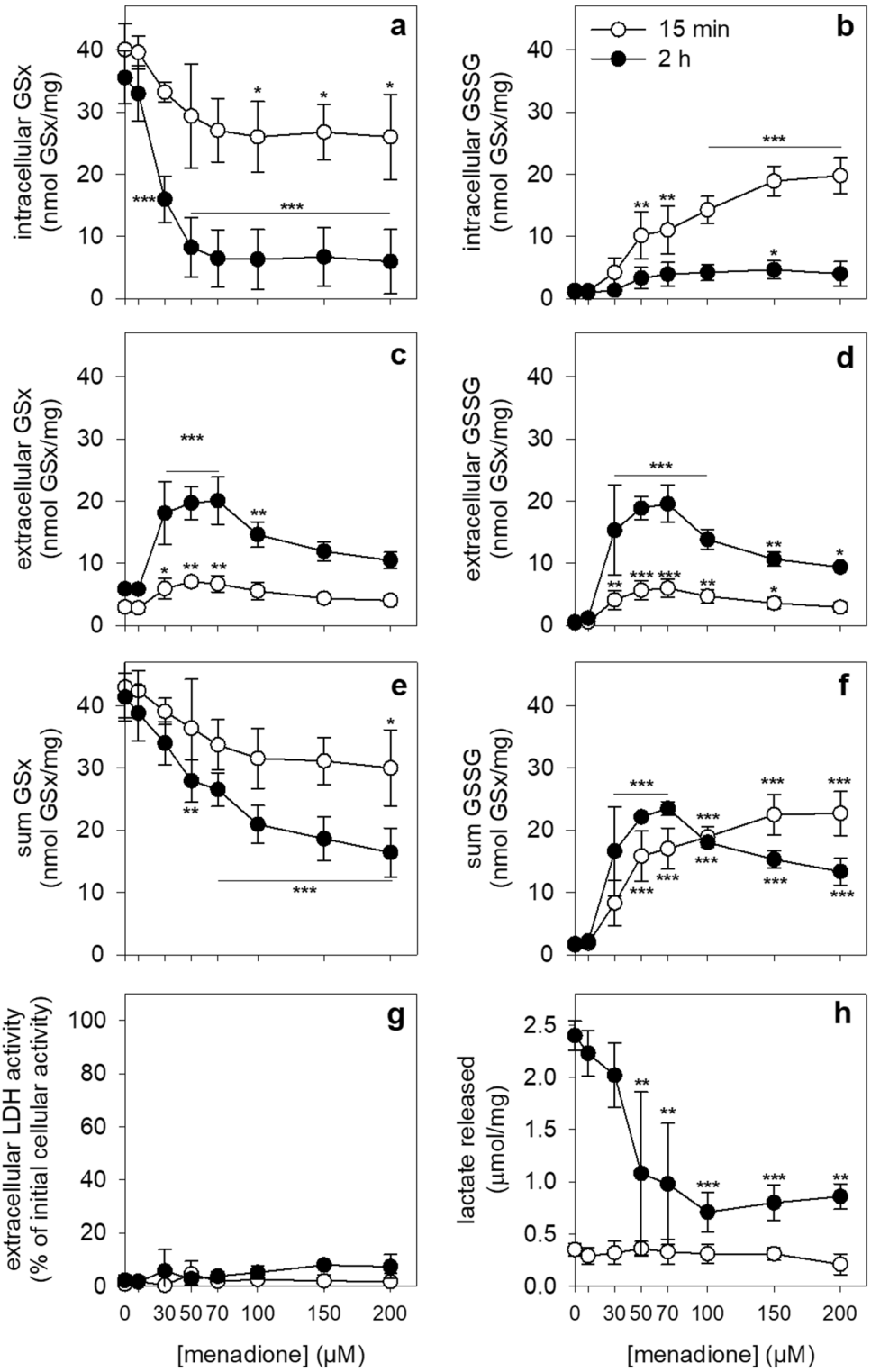


Fig. 4 Concentration-dependent modulation of astrocytic GSH metabolism by menadione. The cultures were incubated without or with the indicated concentrations of menadione for 15 min or 2 h and the intracellular and extracellular contents of GSx (**a, c**) and GSSG (**b, d**), the extracellular LDH activity (**g**) and the extracellular lactate content (**h**) were determined. In addition, the sum of intra- and extracellular GSx (**e**) and GSSG (**f**) were calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 46 ± 5 nmol/mg, the initial cellular GSSG content 1 ± 1 nmol GSx/mg, the initial cellular LDH activity 1622 ± 155 nmol/(min × mg) and the initial protein content 118 ± 25 µg/well. The significance of differences compared to the values obtained for the respective control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Differences between three or more sets of data were tested for significance by analysis of variance (ANOVA) followed by a Bonferroni post hoc test and the statistically significant differences are indicated by asterisks. Significance of differences between two sets of data was analysed by the paired *t* test and the level of significance is indicated by hashes. p values above 0.05 were considered as not significant. The images of stained cells presented in one multipanel figure were obtained in a representative

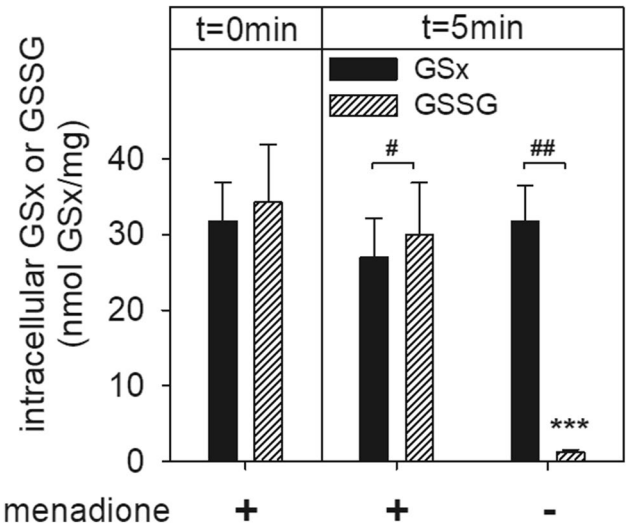


Fig. 5 Reduction of intracellular GSSG after removal of menadione. The cultures were pre-incubated for 5 min with 100 µM menadione (t = 0 min) and subsequently incubated for 5 min without or with 100 µM menadione (t = 5 min) before the cellular contents of GSx and GSSG were determined. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content was 30 ± 3 nmol/mg, the initial cellular GSSG content 0 ± 0 nmol GSx/mg and the initial protein content 99 ± 14 µg/well. The significance of differences compared to the values obtained after the 5 min pre-incubation was analysed by ANOVA and is indicated by asterisks (***$p < 0.001$). Significance of differences between the data obtained for the specific values of GSx and GSSG of one condition was analysed by a paired *t* test and the level of significance is indicated by hashes ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$)

experiment that was reproduced on two independently prepared astrocyte cultures with almost identical outcome.

Results

Menadione-Mediated Toxicity

To test for the potential toxicity of a menadione treatment, cultured primary astrocytes were incubated without or with 10, 30 or 100 µM menadione for up to 6 h and the release of cellular LDH and lactate into the medium was investigated (Fig. 1). Incubations without menadione or with menadione in concentrations of up to 30 µM did not cause any significant increase in extracellular LDH activity (Fig. 1a), in the number of PI-positive cells (Fig. 2b, d, f), in the relative PI fluorescence (Fig. 2i) nor any significant alteration in lactate production (Fig. 1b). In contrast, cells metabolism and membrane integrity were strongly affected during extended incubations of astrocytes with 100 µM menadione, as indicated by the lowered extracellular lactate values found already after 1 h of incubation (Fig. 1b), as well as by the strong increase in extracellular LDH activity, in the number of PI-positive cells and in the relative PI fluorescence of the culture observed after incubation for more than 2 h (Figs. 1a, 2h, i). These data demonstrate that exposure to 100 µM menadione leads to an impairment of astrocytic glucose metabolism and a loss in membrane integrity that are detectable after incubations for 1 h and 4 h, respectively.

Menadione-Induced GSSG Formation and GSSG Export

To test for the potential of menadione to affect the GSH metabolism of astrocytes, the cultures were incubated without or with 10, 30 or 100 µM menadione for up to 6 h. During incubation of astrocytes in the absence of menadione a slow but constant loss in intracellular GSx content was observed (Fig. 3a) that was accompanied by a corresponding increase in extracellular GSx levels (Fig. 3d), resulting in a sum of cellular plus extracellular GSx contents that was almost constant during incubations for up to 6 h and remained almost identical to the initial cellular GSx content of untreated cultures (Fig. 3g). Similar results were observed for astrocytes that had been exposed to 10 µM menadione (Fig. 3). For these conditions, hardly any GSSG was observed in cell lysates and incubation media (Fig. 3b, e, h). In contrast, a concentration- and time-dependent loss in cellular GSx contents and an accelerated appearance of GSx in the medium was observed for cells that had been exposed to 30 µM or 100 µM menadione (Fig. 3a, d). The sum of cellular plus extracellular GSx contents remained almost identical to the values for the control cells (incubation in the absence of menadione) for astrocytes that had been incubated with menadione in concentrations of up 30 µM. In contrast, a significant loss by around 60% of the initial GSx content was observed for cultures that had been incubated with 100 µM menadione for 6 h (Fig. 3g). Incubation of astrocytes with 30 µM and 100 µM menadione caused a mild and strong transient increase, respectively, in the cellular GSSG levels with maximal values that were detectable already after the initial 5 min of incubation (Fig. 3b, c) which was followed by a slow but significant extracellular accumulation of GSSG (Fig. 3e, f). For incubations with 100 µM menadione, almost all the detectable GSx in cells and media represented GSSG (Fig. 3c, f, i), while for cells that had been exposed to 30 µM menadione only around 10% of the cellular GSx but 60% of the extracellular GSx accounted for GSSG (Fig. 3c, f, i). As an incubation with 100 µM menadione for more than 2 h severely impaired membrane integrity (Figs. 1, 2), the percental contribution of GSSG in the GSx values determined was only presented for the initial 2 h of the incubation (Fig. 3c, f, i).

A more detailed analysis of the concentration-dependent potential of menadione to affect the metabolism of

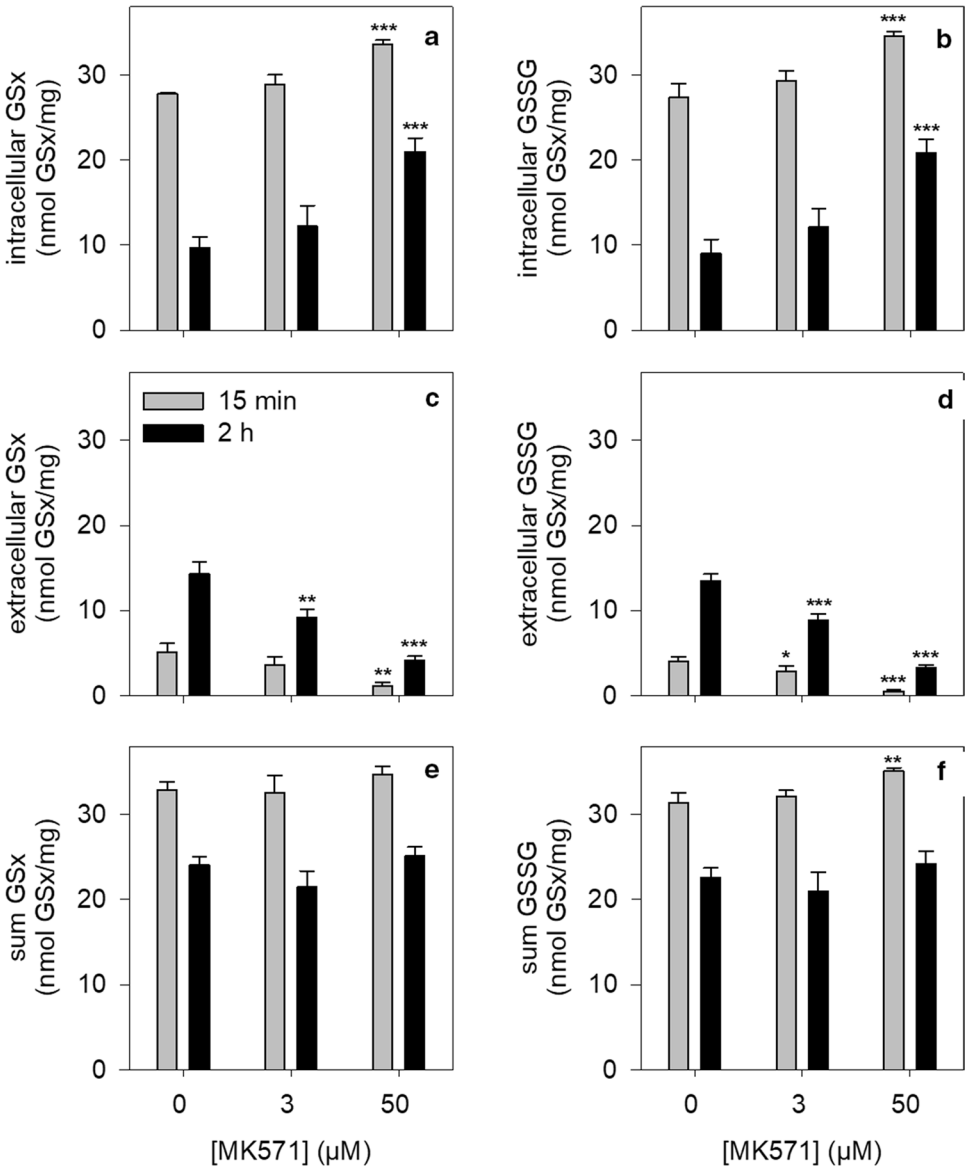


Fig. 6 Inhibition of GSSG export by MK571 in menadione-treated astrocytes. The cultures were incubated for 15 min or 2 h with 100 µM menadione in the absence (0 µM) or the presence of 3 or 50 µM MK571 and the intracellular and extracellular contents of GSx (**a, c**) and GSSG (**b, d**) were determined. In addition, the sum of intra- and extracellular GSx (**e**) and GSSG (**f**) was calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 43 ± 4 nmol/mg, the initial cellular GSSG content 1 ± 0 nmol GSx/mg and the initial protein content 125 ± 19 µg/well. None of the conditions caused any significant increase in extracellular LDH activity (data not shown). The significance of differences compared to the values obtained for the respective control condition (treatment without MK571) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

cultured astrocytes was performed for incubation periods of 15 min and 2 h (Fig. 4). High concentrations of menadione (100–200 µM) caused already within the initial 15 min of incubation a disappearance of cellular (Fig. 4a) and total GSx (Fig. 4e) from the cultures as well as a rapid appearance of GSSG in the cells (Fig. 4b), while little amounts of extracellular GSx and GSSG were determined after 15 min incubation (Fig. 4c, d). However, after 2 h of incubation the cells were almost completely depleted of GSx and GSSG after incubation with menadione in concentrations above 30 µM (Fig. 4a, b) and large amounts of GSSG were determined in the medium (Fig. 4c, d). None of the conditions applied significantly increased extracellular LDH activity (Fig. 4g), demonstrating that viable cells had been investigated. After 2 h of incubation with menadione in concentrations of 50 µM or higher a significant decline by about 70% in the accumulation of extracellular lactate was observed, while after the initial 15 min of incubation no alteration in lactate release was observed even for cultures that had been exposed to 200 µM menadione (Fig. 4h).

Reversibility of the Menadione-Induced GSSG Accumulation

To test for the potential of menadione-treated astrocytes to regenerate the initial very low cellular GSSG to GSx ratio, the cultures were pre-incubated with 100 µM menadione for 5 min. Subsequently, the menadione-containing IB was removed and the cells were incubated for 5 min either in fresh menadione-containing IB or in menadione-free IB. During the short pre-incubation with menadione cellular GSH was rapidly oxidized to GSSG as demonstrated by the observation that the

GSx values determined represented exclusively GSSG (Fig. 5). This high ratio of GSSG to GSx was maintained in cells that were incubated for additional 5 min with menadione (Fig. 5). In contrast, 5 min after removal of menadione cellular GSSG accounted only to 4% of the GSx values determined (Fig. 5), demonstrating rapid reduction of cellular GSSG after removal of menadione.

Inhibition of Menadione-Induced GSSG Export by MK571

Cellular GSSG is exported from viable astrocytes by Mrp1 [19, 21]. To investigate whether the extracellular GSSG accumulation observed for menadione-treated astrocytes is mediated by Mrp1, the Mrp1-inhibitor MK571 [17, 19] was applied. After application of 100 µM menadione for 15 min or 2 h the viability of the cells was not affected as demonstrated by the absence of any increase in extracellular LDH activity (data not shown) and the cellular and extracellular GSx values represented almost exclusively GSSG for all conditions investigated (Fig. 6). The presence of MK571 lowered in a concentration-dependent manner already during the initial 15 min of incubation with 100 µM menadione the loss in cellular GSx and GSSG (Fig. 6a, b) and the extracellular accumulation of GSx and GSSG (Fig. 6c, d). The extent of the inhibitory effect of MK571 on GSSG export was found more pronounced after 2 h of incubation (Fig. 6a–d). In contrast, neither the sum of cellular plus extracellular GSx or GSSG nor the time-dependent menadione-induced disappearance of around 30% of the initial cellular GSx content between 15 min and 2 h of incubation was affected by the presence of MK571 (Fig. 6e, f).

ROS Production in Menadione-Treated Astrocytes

Menadione has been reported to cause ROS formation in cells [39] and cultured astrocytes [29, 30]. In order to investigate whether also under the conditions used here the application of menadione causes rapid ROS production in astrocytes, the presence of ROS was detected for the cells by rhodamine123 staining. After incubation of cultured astrocytes in the absence of menadione only little rhodamine123 staining was observed (Fig. 7b), while already after a 5 min exposure of the cells to menadione a concentration-dependent increase in the number of ROS-positive cells was observed (Fig. 7f, k, o, s) with a ROS staining detectable for the majority of cells in cultures that had been treated with 100 µM menadione (Fig. 7s).

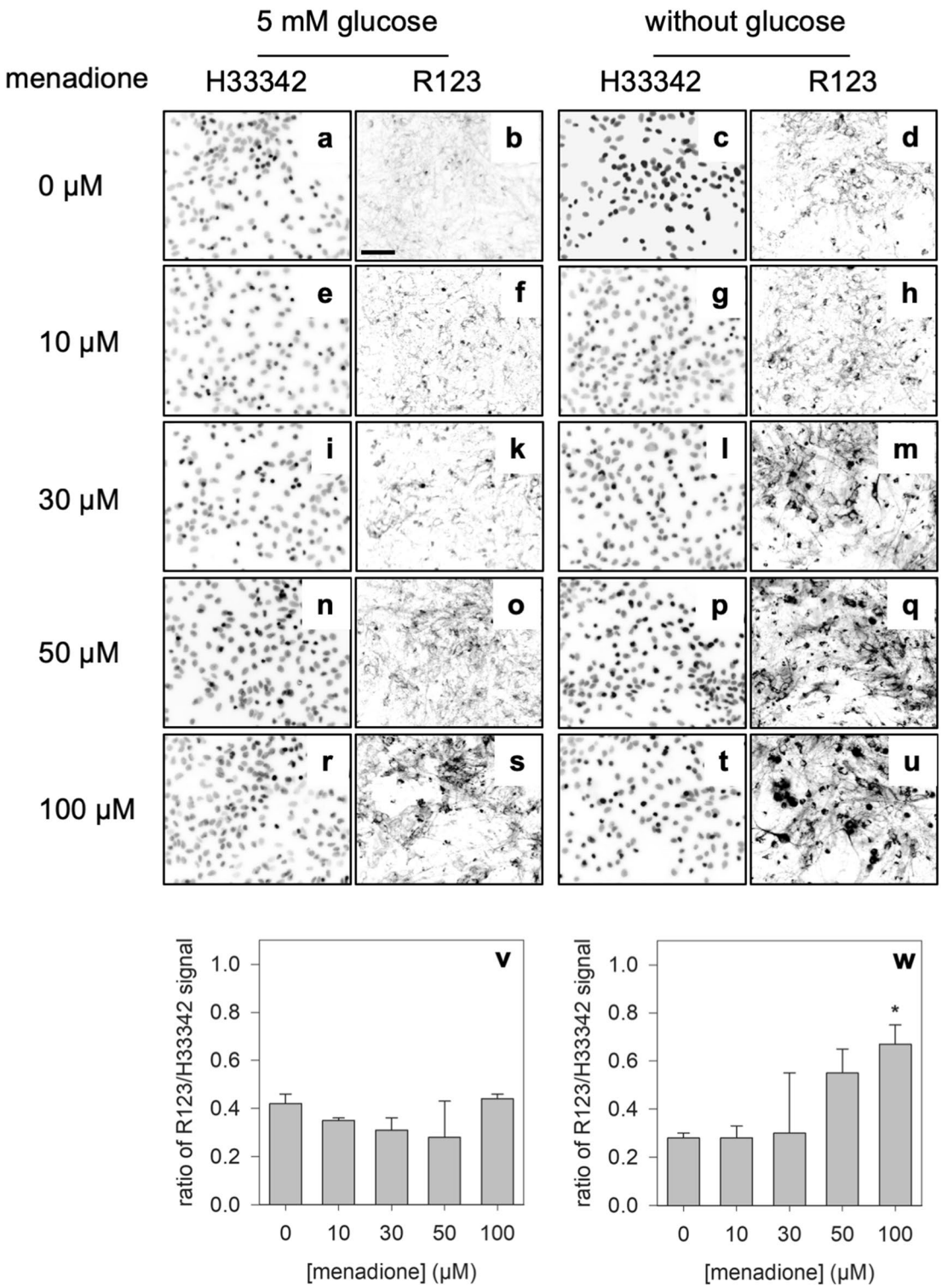
Modulation of Menadione-Induced Formation of ROS and GSSG by the Absence of Glucose

Availability of glucose is important to regenerate via the pentose phosphate pathway NADPH that is required for antioxidative defence [40, 41]. To test whether the menadione-mediated ROS generation and GSH oxidation may be affected by the absence or the presence of glucose and its metabolites, the cells were deprived of intracellular glucose by pre-incubation for 20 min in glucose-free IB (or as glucose control in IB containing 5 mM glucose) and were then incubated with 100 µM menadione in the absence or the presence of 5 mM glucose.

Glucose deprivation did to some minor extent increase the number of ROS-positive cells (Fig. 6b, d) but did not affect the basal cellular and extracellular levels of GSx and GSSG (Fig. 8) in cultures that had not been exposed to menadione. Application of menadione to glucose-deprived and glucose-fed astrocytes caused a concentration-dependent appearance of ROS (Fig. 7) and a concentration-dependent accumulation of intracellular and extracellular GSSG (Fig. 8). However, in glucose-deprived cells the ROS production was found more pronounced (Fig. 7h, m, q, u) than in glucose-fed cells (Fig. 7f, k, o, s). For example, ROS production was already clearly detectable for incubations with 30 µM menadione in glucose-deprived cells (Fig. 7m), but not in glucose-fed cells (Fig. 7k). Also, the quantified and normalized intensity of the ROS staining was found increased with the concentration of menadione applied in glucose-deprived cells (Fig. 6w), but not in glucose-fed cells (Fig. 6v). Similarly, 50 µM or 100 µM of menadione had to be applied to glucose-fed cells to cause a strong increase in cellular GSSG levels, while already a treatment with 10 µM menadione was sufficient to significantly increase cellular and extracellular contents of GSSG in glucose-deprived cells (Fig. 8). The effects observed for a treatment with menadione on the cellular and extracellular levels of GSx and GSSG were for almost every concentration of menadione applied significantly stronger for glucose-deprived cells compared to the respective incubation in the presence of glucose (Fig. 8).

Test for an Involvement of NQO1 in the Menadione-Induced GSH Oxidation

Menadione is a substrate of the enzyme NQO1 [4]. In order to test whether NQO1 is involved in the observed menadione-induced GSH oxidation in astrocytes, the consequences of a menadione exposure to astrocytes in the absence or the presence of the highly potent NQO1 inhibitor dicoumarol [5] was investigated. The presence of 30 µM dicoumarol did not prevent but rather slightly increased the menadione-induced appearance of GSSG in the cultures and did also not prevent the cell toxicity observed after 4 h of menadione treatment (Table 1). Also, the rapid occurrence of ROS in menadione-treated astrocytes was not prevented by the presence of dicoumarol (data not shown). However, the presence of dicoumarol for 15 min caused an increase in the cellular levels of GSx



and GSSG and partially prevented the acute (15 min) extracellular accumulation of GSSG (Table 1), consistent with the recently described potential of dicoumarol to inhibit Mrp1-mediated transport processes [32]. The high extracellular contents of GSx and GSSG determined after 4 h of incubation for both conditions are most likely a consequence of a severely impaired membrane integrity, as demonstrated by the elevated activity of extracellular LDH determined for incubations with menadione in the absence or the presence of dicoumarol (Table 1).

◂**Fig. 7** Effects of the absence or the presence of glucose on the menadione-induced ROS formation. Astrocyte cultures were pre-incubated for 20 min without or with 5 mM glucose and then incubated without or with the indicated concentrations of menadione for 5 min in the absence or the presence of 5 mM glucose. After the incubation, the presence of ROS was visualized by monitoring rhodamine123 (R123) fluorescence. Hoechst 33342 staining was applied to visualize the total number of cell nuclei present. The scale bar in panel b represents 50 µm and applies to all panels. Panels v and w show the relative R123 fluorescence (ratio of the R123–H33342 fluorescence intensity signals) calculated for cells that had been incubated in the presence of 5 mM glucose (v) or in the absence of glucose (w). The quantitative data are means ± SD of results that were obtained in experiments performed on three independently prepared cultures. The significance of differences compared to the values obtained for the respective control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$)

Discussion

Exposure of cultured astrocytes to menadione caused a rapid concentration-dependent increase in cellular ROS and GSSG within minutes that was followed by an impairment of cellular lactate production and finally by a loss in cell membrane integrity. Menadione has previously been applied to cultured astrocytes to induce oxidative stress [26–28]. The menadione-induced ROS production and toxicity observed in our study for cultured astrocytes that had been exposed to high concentrations of menadione is consistent with literature data [29–31]. Menadione is a membrane-permeable molecule that easily penetrates the intact cell membrane of viable astrocytes [42] and leads to rapid ROS formation in the cells. This ROS formation is likely to cause oxidative stress in the cells by oxidation of cellular molecules such as GSH even if the cell membrane is intact, consistent with the reported early damage of mitochondrial DNA in intact astrocytes after menadione exposure [28, 43].

Application of 100 µM menadione caused a rapid formation of ROS and a rapid oxidation of GSH to GSSG in the cells. As GSH is able to reduce various ROS which leads to the generation of GSSG [6, 8, 10], it is assumed that the rapid accumulation of GSSG in astrocytes after application of higher concentrations of menadione is the direct consequence of GSH-mediated ROS detoxification. An impairment of antioxidative enzymes by menadione application such as superoxide dismutase and glutathione peroxidase has not been found for menadione-treated astrocytes [43]. Also, a potential contribution of an inhibition of glutathione reductase in the rapid accumulation of cellular GSSG in menadione-treated cells appears unlikely, as the initial high GSH to GSSG ratio of untreated astrocytes was fully restored within 5 min after removal of menadione following a 5 min exposure to 100 µM menadione.

The extent of adverse consequences of a menadione treatment of cultured astrocytes depends strongly on the concentration of menadione applied. While the cells could deal very well with an exposure to menadione in concentrations of up to 30 µM, the application of 100 µM menadione for more than 2 h severely compromised cell viability. The low levels of ROS production and cellular GSSG accumulation as well as the low toxic potential of menadione in concentrations of up to 30 µM is most likely a consequence of the highly efficient antioxidative defence system of astrocytes [6, 44] which prevented the accumulation of detectable amounts of ROS and a substantial cellular GSSG accumulation under the conditions investigated. This view is supported by the observation that menadione caused already in concentrations of 10 µM detectable ROS production and substantial GSSG oxidation in glucose-deprived astrocytes, consistent with the need of astrocytes for pentose-phosphate pathway (PPP)-derived NADPH for efficient GSSG reduction via glutathione reductase [41, 45].

The menadione-mediated ROS production in astrocytes may involve a direct chemical GSH oxidation by menadione, but also the one-electron reduction of menadione by cytochrome P450 monooxygenases to the unstable semiquinone that causes subsequently cellular ROS production [4, 29, 46]. In contrast, the cytosolic enzyme NQO1 which catalyses the obligatory two-electron reduction which avoids the formation of a semiquinone radical [4, 5] appears to be not involved in the menadione-induced ROS production, as the NQO1 inhibitor dicoumarol was unable to prevent ROS formation and GSSG accumulation in menadione-treated astrocytes. However, it should be stressed here that dicoumarol lowered the export of GSSG from menadione-treated astrocytes consistent with recent literature data [32], demonstrating that dicoumarol cannot be considered anymore as a selective inhibitor of NQO1.

An additional consequence of an application of menadione in concentrations above 30 µM was a disappearance of detectable GSx (sum of GSx in cells plus medium) from the cultures that accounted after 2 h of incubation to around 40% of the initial cellular levels of GSx. This loss in GSx is consistent with recent literature data [32] and is most likely caused by the chemical or enzyme-catalyzed formation of a menadione-GSH conjugate [2, 24] that cannot be detected by the enzymatic cycling assay used for the quantification of GSx.

Application of menadione in a concentration of 100 µM caused a rapid and transient accumulation of GSSG in astrocytes. Maximal cellular GSSG values were found already after 5 min of incubation and the cellular GSSG accounted after 5 min to around 70% of the cellular GSx content, demonstrating that under the conditions used glutathione reductase was not able to immediately reduce the GSSG generated from GSH during ROS detoxification. The loss in cellular GSSG during the subsequent longer incubation was accompanied by the accumulation of GSSG in the incubation medium of viable cells, suggesting that the intracellular

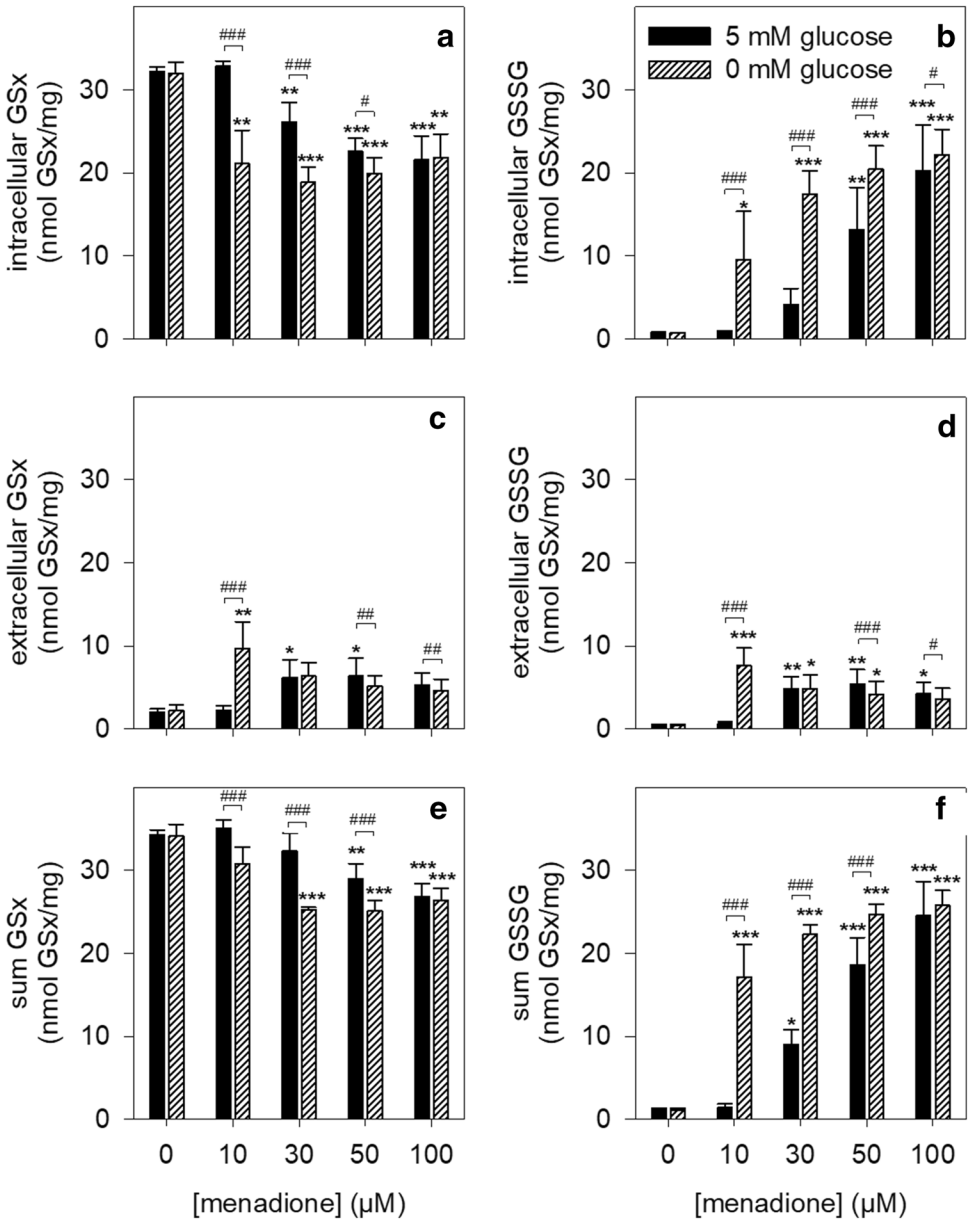


Fig. 8 Effects of the absence or the presence of glucose on the menadione-induced GSSG formation. Astrocyte cultures were pre-incubated for 20 min without or with 5 mM glucose and then incubated without or with 10, 30, 50 or 100 μM menadione for 15 min in the absence or the presence of glucose. Afterwards, the intracellular and extracellular contents of GSx (**a, c**) and GSSG (**b, d**) were determined. In addition, the sum of intra- plus extracellular GSx (**e**) and GSSG (**f**) were calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 36 ± 4 nmol/mg, the initial cellular GSSG content 1 ± 0 nmol GSx/mg and the initial protein content 125 ± 19 μg/well. None of the conditions caused any significant increase in extracellular LDH activity (data not shown). The significance of differences compared to the values obtained for the respective control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Differences between values obtained for incubations in the absence and in the presence of glucose were analysed by a paired *t* test and the level of significance is indicated by hashes ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$)

GSSG was exported from the cells via a transporter. Indeed, MK571 and dicoumarol, known inhibitors of Mrp1-mediated transport processes [17, 21, 32] inhibited the loss of cellular GSSG and the extracellular accumulation of GSSG in menadione-treated astrocyte cultures, suggesting that Mrp1 is mediating the observed GSSG export. Substantial extracellular GSSG accumulation was already observed for astrocytes that had been exposed to 30 μM menadione, although only little cellular accumulation of GSSG was detected for this condition which did not exceed 10% of the cellular GSx content. However, as the GSH concentration in cultured astrocytes is around 8 mM [7] the intracellular GSSG concentration will be around 400 μM even if GSSG accounts for only 10% of the cellular GSx concentration. At this GSSG concentration efficient export of GSSG via Mrp1 has to be expected, as this transporter has a K_M value for its substrate GSSG of 93 μM [18].

Menadione in a concentration above 30 μM lowered also the glycolytic lactate production of cultured astrocytes. As this effect was only observed after more than 15 min of incubation, the lowered lactate production is likely to be a consequence of the initial rapid ROS formation and GSSG accumulation in menadione-treated astrocytes. A reduction in the rate of glycolytic lactate production has also been reported for astrocytes that were exposed to chronic hydrogen peroxide stress, a condition that also caused a rapid accumulation of GSSG in astrocytes [36]. The high ratio of cellular GSSG to GSH found under such stress conditions is likely

Table 1 Modulation by dicoumarol of the menadione-mediated effects on astrocytes

Parameter investigated	Units	Menadione		Menadione + dicoumarol	
		15 min	4 h	15 min	4 h
Extracellular LDH	% Initial cellular activity	0.9 ± 0.8	30.9 ± 6.2	0.5 ± 0.4	24.6 ± 9.7
Intracellular GSx	nmol GSx/mg	30.3 ± 1.8	1.0 ± 0.5	37.7 ± 0.8$^{\#\#}$	2.4 ± 0.1$^{\#}$
Intracellular GSSG		30.7± 6.9	1.5 ± 0.5	40.6 ± 6.0$^{\#}$	2.9 ± 0.3$^{\#}$
Extracellular GSx		5.9 ± 0.6	21.2 ± 1.9	2.0 ± 0.5$^{\#}$	22.5 ± 4.3
Extracellular GSSG		5.3 ± 0.3	19.9 ± 2.1	1.9 ± 0.7$^{\#}$	22.7 ± 5.0
Sum GSx		36.2 ± 1.2	22.1 ± 2.3	39.7 ± 1.1$^{\#\#}$	24.9 ± 4.3
Sum GSSG		36.0 ± 6.6	21.4 ± 2.4	42.5 ± 6.5$^{\#}$	25.6 ± 5.2

Cultured astrocytes were incubated with 100 µM menadione in the absence or the presence of 30 µM dicoumarol for 15 min or for 4 h before the extracellular LDH activity as well as the intracellular and extracellular contents of GSx and GSSG were determined. In addition, the sum of intracellular plus extracellular GSx and of intracellular plus extracellular GSSG were calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 49 ± 4 nmol/mg and the protein content was 121 ± 15 µg/well. The initial cellular LDH activity (100%) was determined as 2163 ± 150 nmol/(min × mg). The significance of differences between data obtained for treatments without and with dicoumarol was analysed for the respective time points by the paired *t* test and the level of significance is indicated by hashes ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$)

to trigger a redox switch which re-directs the metabolism of glucose from glycolysis into the oxidative parts of the PPP in order to facilitate re-generation of NADPH that is needed for antioxidative defence [41, 47, 48].

Oxidative stress is frequently applied to cultured brain cells by exposure to hydrogen peroxide [49, 50]. As especially astrocytes but also other brain cell types in culture are very efficient to dispose applied hydrogen peroxide [44] the extent of intracellular GSSG accumulation after application of a peroxide is low and is restricted to the time period required for the cellular detoxification system to remove the applied peroxide which immediately enables glutathione reductase to regenerate the initial very high GSH to GSSG ratio. In contrast, during exposure to 100 µM menadione a rapid and extensive ROS formation was observed that was accompanied with a quick and extensive oxidation of the cellular GSH to GSSG within minutes. As the redox cycling of menadione has the potential to generate continuously ROS for at least 1 h [26], the ROS-mediated GSH oxidation continues and cellular GSSG accumulation cannot be efficiently prevented by glutathione reductase. This makes an cellular export of GSSG via Mrp1 a preferred option to lower the cellular concentration of GSSG and thereby the high ratio of GSSG to GSH. Thus, the menadione-induced rapid and extensive increase in cellular GSSG concentration can be used as valuable experimental approach to investigate consequences of oxidative stress on the GSH metabolism in astrocytes and in particular to investigate GSSG export processes as recently described [32].

In conclusion, application of menadione to cultured astrocytes triggers a rapid concentration-dependent ROS formation and GSH oxidation which is followed by Mrp1-mediated export of GSSG. This confirms the strong potential of menadione to cause oxidative stress in cultured brain cells by a NQO1-independent process. The experimental setup used allows to investigate various aspects of menadione-induced oxidative stress and defines the conditions suitable to study ROS formation, GSH oxidation and Mrp1-mediated GSSG export as well as ROS-induced toxicity in cultured brain astrocytes after menadione exposure.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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